Development of Inducible Constructs and Stable Cell Lines for Cbl Expression

A Senior Honors Thesis

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of The Ohio State University

by
Solomon Abay

The Ohio State University
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Project Advisor: Dr. Nancy Lill, Department of Pathology

The Ohio State University Medical Center
Department of Pathology
Columbus, Ohio

CERTIFICATE OF APPROVAL

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SENIOR HONORS THESIS

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This is to certify that the Senior Honors thesis of

Solomon Abay

has been approved by the Examining Committee for the thesis requirement for graduation *with research distinction* at the June 2010 graduation.

Thesis Committee: _____
Nancy Lill, Thesis Supervisor

_____
Marshall Williams

_____
James Waldman

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I would also like to thank Dr. Sarah Cole and Jennifer Zhang for all of their help. As previous and current lab managers, respectively, of the Lill laboratory, their efforts to manage the day-to-day operations of the lab were detrimental to this project. In addition to the lab managers, I would be remiss if I failed to thank my fellow undergraduates who have worked tirelessly to ensure the smooth operation of the lab at all times. These include Rajeev Sachdeva, Amanda Townsley, and especially Aimee Schmenck. It was through their combined efforts that my project was able to carry on unabated.

Finally, I wish to thank my family for their unwavering love and support. Though they would sometimes find it difficult to understand why I was spending so much of my free time working in a lab, they never stopped encouraging me to pursue my goals.

Abstract

The E3 ubiquitin ligase Cbl plays a vital role in the trafficking of ligand-bound EGF-R through the endocytic pathway to its subsequent degradation. The RING finger tail region of the Cbl protein is thought to be critical to this process. Previous studies demonstrated that amino acid substitutions at position 431 or 434 of Cbl can disrupt the normal trafficking of EGF-R. Despite these previous findings regarding trafficking, the role of these mutations on EGF-R signaling has yet to be examined. In this study, we aim to begin to test the effects on signaling of the V431A and F434A mutations in a cellular context by developing pertinent plasmid constructs and generating stable, inducible cell lines for the signaling studies.

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Chapter I: Introduction

One characteristic of many cancers is cellular hyperproliferation associated with an abnormal increase in the activity of the epidermal growth factor receptor (EGF-R) (5, 6). Balanced regulation of this receptor tyrosine kinase is crucial for normal growth and development: while an absence of EGF-R signaling is lethal to knockout mice (10, 15), excessive signaling can cause cancer to develop (3). Research into the mechanisms used by normal cells to regulate EGF-R may reveal new molecular targets for novel anticancer therapies.

Regulated receptor trafficking and degradation are required to maintain normal EGF-R levels and signaling. One mechanism of signaling suppression involves EGF-R trafficking through the endocytic pathway to lysosomes, where the receptor is degraded. In this pathway, ligand-activated EGF-R dimerizes and is ubiquitinated at the cell surface by the E3 ubiquitin ligase Cbl. Ubiquitinated receptors are internalized on vesicles that fuse with early endosomes. At this stage, EGF-R is either recycled to the cell surface or sorted onto the luminal membranes of multivesicular bodies (MVBs), which are more mature forms of the sorting endosomes. Delivery to the endosome lumen physically separates activated receptors from their cytosolic signaling partners. It also targets the ubiquitinated proteins for lysosomal delivery. This results in the destruction of the receptors and their associated proteins. Lysosomal degradation is a definitive mechanism for EGF-R signaling termination (1, 7, 13).

There are three different types of Cbl proteins found in humans, each containing several different functional domains. The region of Cbl most significant to this project is an evolutionarily conserved region comprising the first 436 amino acids (Figure 1).

Domains included in this conserved region are the TKB domain, the linker (L) region, the RING finger (RF) domain, and the RF tail -- which is the focus of this project. The RF tail, made up of less than 20 amino acids, is vital to the proper functioning of the entire Cbl protein. Truncation of Cbl to amino acid 436 results in a functional protein. Removal of three amino acids has been shown to result in a nonfunctional Cbl protein. As mentioned above, Cbl belongs to the family of proteins known as E3 ubiquitin ligases. During the process of ubiquitination, the E3 ligase acts as an adaptor molecule for transfer of an ubiquitin molecule to the protein substrate being targeted for degradation (Figure 2).

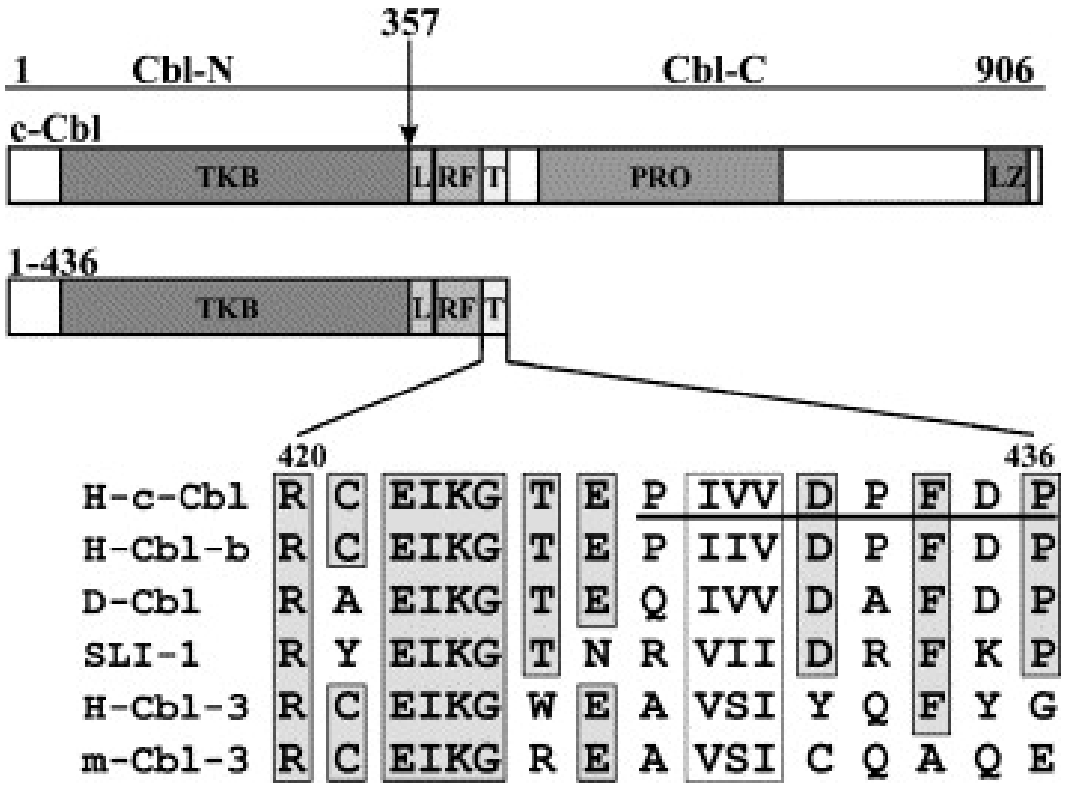


Figure 1. Graphic representation of the Cbl protein.

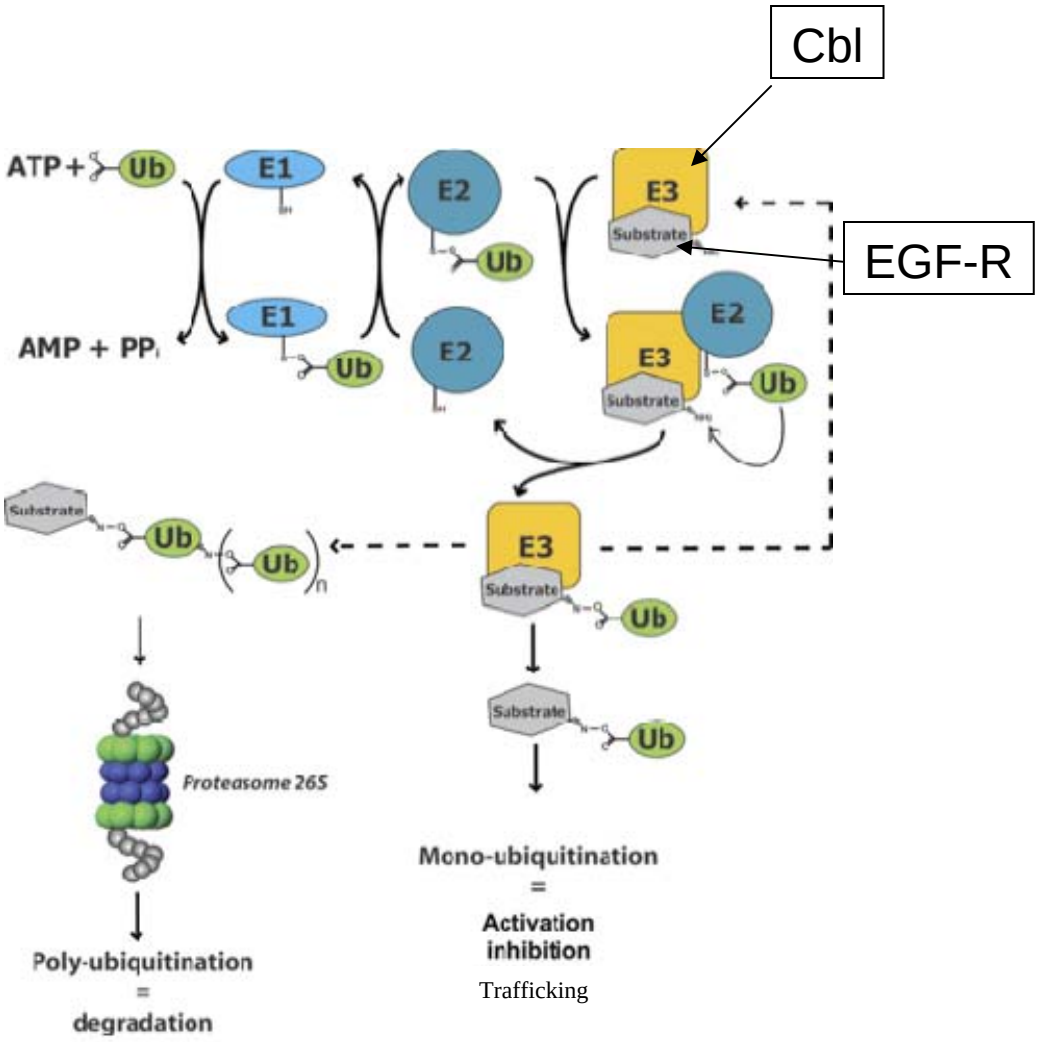


Figure 2. The ubiquitination pathway.

The Lill laboratory has been investigating the nature of molecular complexes that regulate EGF-R endocytic trafficking downstream of internalization. Their recently published work revealed important roles for the proteins Cbl and hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) in controlling a newly identified EGF-R trafficking checkpoint (12, 15). At this checkpoint, EGF-R-associated Cbl enhances the tyrosine phosphorylation and ubiquitination of the Hrs protein. Hrs is a critical regulator of endosome docking and fusion (14). Hrs phosphorylation causes Hrs degradation, removing it from endosomal EGF-R/Cbl complexes. As a result, Hrs no longer inhibits the fusion and maturation of endosomes, allowing EGF-R/Cbl complexes to proceed to the endosomal lumen.

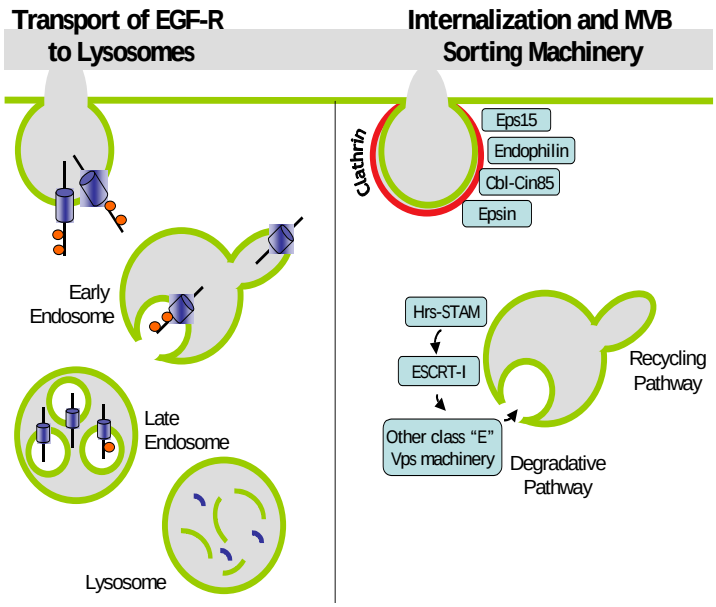


Figure 3. The endocytic pathway leading to multivesicular body formation. (Left) Ligand-activated EGF-R is: ubiquitinated at the cell surface; internalized on vesicles that fuse with early endosomes; and either recycled or sorted onto lumenal membranes that are internalized from endosomal limiting membranes. Lumenal vesicle formation, initiated at early endosomes, is complete at late endosomes (multivesicular morphology). Lysosomal delivery allows for destruction of internal membranes and their cargo. (Right) Proteins important for EGF-R trafficking. Eps15 and Epsin bind ubiquitin and are involved in EGF-R internalization. Endosomal ubiquitin-binding receptors (Hrs-STAM and ESCRT-I, which contains the ubiquitin-binding protein Tsg101) bind EGF-R and direct it to MVB internal membranes.

Structure-function studies in the Lill laboratory have shown that the RF tail is essential for Hrs regulation. Single amino acid substitutions of V431A and F434A in the

RF tail domain result in Cbl proteins that cannot regulate Hrs properly. The V431A mutant blocks activated EGF-R at the cell surface, so it never encounters Hrs on endosomes. The F434A mutant has a more interesting phenotype: it allows receptor/Cbl complexes to move to endosomes, where Hrs phosphorylation takes place. However, this phosphorylated Hrs does not get degraded. Instead, it remains on the endosomes and blocks endosome fusion from occurring. The Cbl F434A mutant therefore represents a unique tool for the molecular analysis of how normal (wild type) Cbl controls the fusion checkpoint to direct activated receptors to luminal vesicles and lysosomal degradation. If we can determine why wild type Cbl is functional and F434A is not, we will understand a new regulatory step in the EGF-R degradation process.

The primary focus of this project was to develop experimental reagents to address the question "What impact does RF tail mutation have on EGF-R signaling due to the deregulation of the V431- or F434-dependent trafficking checkpoint?"

Chapter II: Methods

Plasmids. The parent construct pTRE-GFP-Cbl-N was generated by first carrying out a mutagenic PCR procedure using the lab-supplied plasmid pcDNA3-GFP-Cbl-N and the Platinum Taq HiFi Polymerase, dNTPs, $MgSO_4$, and buffer from Invitrogen. A lab-supplied stock of the primer IA-193 (5' – GCGGTACCATGAGTAAAGGAGAAGAA CTCTTC – 3'; Integrated DNA Technologies, IDT) was utilized as the upstream primer in order to introduce a KpnI restriction site just upstream of the GFP gene. For the downstream primer, a new oligonucleotide primer, OH-019 (5' – CGTCTAGACTATCA GGGAGTTGGTTCACATAAGCC – 3'; IDT), was created. The resulting PCR product was characterized by electrophoresis on a 1.0% agarose/TAE gel. The size of 1.8 kbp

confirmed the success of the procedure. The PCR product was then digested with KpnI and XbaI, as was pTRE, allowing for ligation of the insert (GFP-Cbl-N) to the linearized vector (pTRE), using T4 DNA ligase and corresponding buffer (BioLabs). The DNA constructs were then used to transform competent JM109 *E. coli* cells, followed by plating on LB-ampicillin agar. After purification of DNA prepared from the resulting colonies using a Maxiprep kit (QIAgene), the DNA was digested with KpnI and XbaI and then separated on a 1.0% agarose/TAE gel for characterization of the parent construct, pTRE-GFP-Cbl-N.

This was followed by sequential digestion and insertion of the alternate Cbl sequences (wt, V431A, and F434A) using the restriction enzymes BamHI and XbaI, followed by ligation, transformation, and purification of DNA, as mentioned above and in the Results section.

Mammalian cell lines, their passaging and maintenance. This study utilized HEK 293-TRE Tight-Tet-ON Advanced cells (Clontech). HEK 293 is an immortalized human embryonic kidney cell line of epithelial morphology. The cells express very low levels of EGF-R and related receptor tyrosine kinases; by introducing expression constructs into them, we can evaluate the impact of co-expressed proteins on EGF-R homodimers, which are the normal targets for Cbl. The HEK 293 cell model system has been proven by the Lill lab to differentiate activities of wild type and mutant Cbl proteins in assays of EGF-R ubiquitination, downregulation, degradation, and signaling (9, 12, 15). By moving our studies into the HEK 293-TRE tight-Tet-ON Advanced cells, we have maintained our experimental system while adding the ability to express the Cbl protein of interest only when it is desired.

The parental and derived HEK 293-TRE tight-Tet-ON Advanced cell lines were passaged regularly at subconfluence, using trypsin/EDTA solution and an established laboratory protocol. The cells were maintained in DMEM containing fetal bovine serum (10%), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 units/mL penicillin-streptomycin and 20 mM HEPES, in an atmosphere containing 5% CO_2.

Growth of bacteria and large-scale plasmid preparation. Frozen glycerol stocks of bacteria carrying expression constructs for EGF-R and constitutively expressed GFP-tagged wild type (wt), V431A, F434A, and –N mutant Cbl were reported previously (16) and available in the laboratory. Large-scale bacterial cultures were grown in the presence of ampicillin. The bacteria were then processed for plasmid preparation using the QIAGEN Maxiprep system. Newly developed pTRE-GFP and pTRE-GFP-Cbl-wt, -V431A, -F434A, and -Cbl-N plasmids were propagated and purified identically.

Transfection of Mammalian Cells. Established techniques were used to introduce the mammalian expression constructs into the cells to be tested. HEK 293-TRE tight-Tet-ON Advanced cells were transfected using a modification of the calcium phosphate precipitation technique (4). DNA precipitates/complexes were normalized for total DNA and promoter input. Transfections resulted in either transient expression of the proteins of interest for short-term assays or in the development of clonally derived inducible cell lines. This was important, as stable cell lines continuously expressing wild type Cbl utilize undefined compensatory mechanisms to override Cbl's suppression of RTK signaling, thus confounding readouts of Cbl's impact on cell signaling and growth, and perhaps also receptor trafficking (2, 11).

Chapter III: Results

A. Generation of constructs for inducible mammalian expression of GFP and GFP-Cbl fusion proteins.

Cbl RF tail residues V431 and F434 are critical for EGF-R down-regulation and degradation. We wished to determine whether these residues influence receptor signaling in a cellular context. Over-expression of wild type Cbl has been shown to select for the outgrowth of cells that have bypassed Cbl-regulated signal suppression (7, 11). To avoid this problem, tetracycline-inducible constructs encoding GFP or GFP-tagged wildtype, V431A, F434A, or the transforming truncation mutant Cbl-N were developed as specified in the Methods section.

The original intention was to generate five plasmid constructs (Figure 4) by carrying out digestions of the donor plasmids pcDNA3-GFP-2xStop, GFP-Cbl-wt, -N, -V431A, and -F434A using the restriction enzymes HindIII and XbaI, followed by insertion of the different GFP-Cbl DNA fragments into the like-digested pTRE-tight cloning vector(Figure 5). The general design of the expected insert donor constructs is shown in Figure 6. But upon attempting this digestion/insertion strategy, I discovered that the Cbl coding sequence possesses additional restriction sites for the enzyme HindIII. This problem was not observed in the pcDNA3-2xStop plasmid, which possesses only a GFP insert and no Cbl coding sequence. Therefore, the pTRE-GFP construct (Figure 7) was generated using the above-mentioned strategy, while the pTRE-GFP-Cbl-wt, -N, -V431A, and –F434A constructs were not (Figure 8).

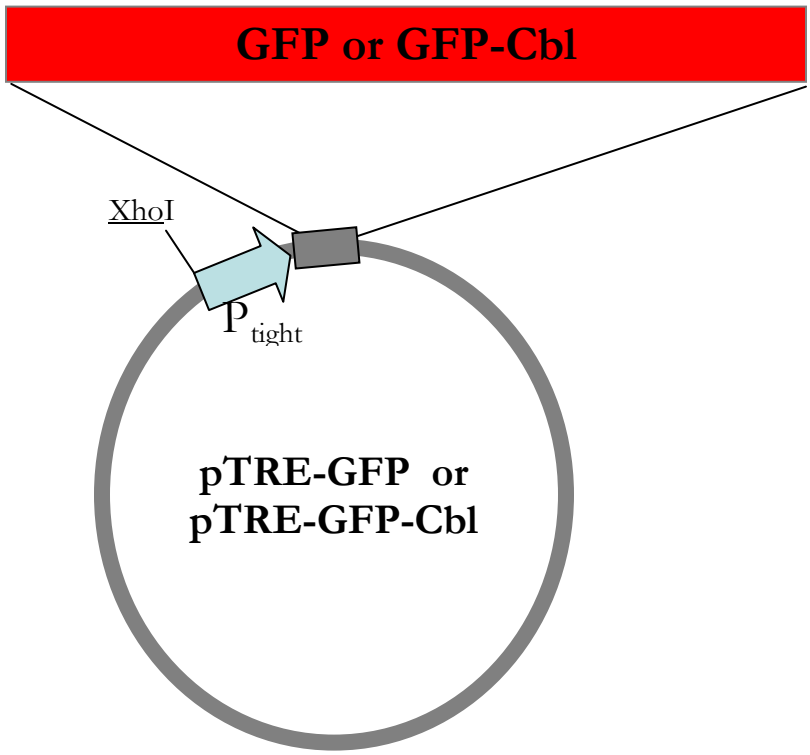


Figure 4: Basic composition of inducible constructs to be generated. The goal was to generate five different plasmid constructs using the pTRE-tight cloning vector. The inserts, GFP or GFP-Cbl, would be inserted downstream of the P_{tight} inducible promotor.

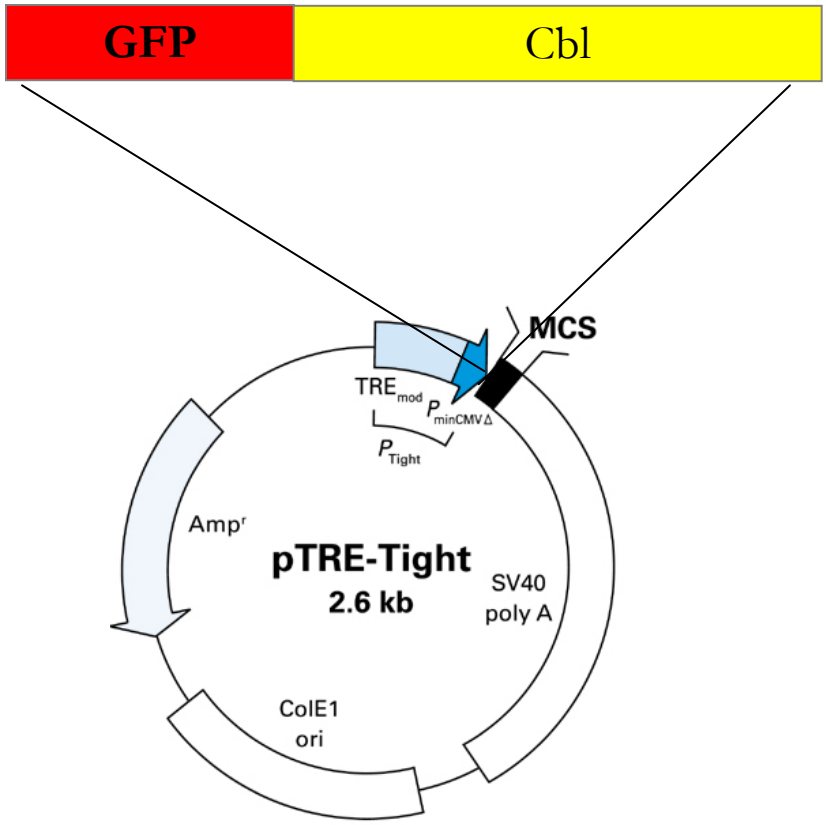


Figure 5: Specific details of the cloning vector, pTRE-Tight, and the sequences to be cloned. Five different inserts were cloned into the vector pTRE-Tight. These included: GFP, GFP-Cbl-N, GFP -Cbl-wt, GFP-Cbl-V431A, and GFP-Cbl-F434A.

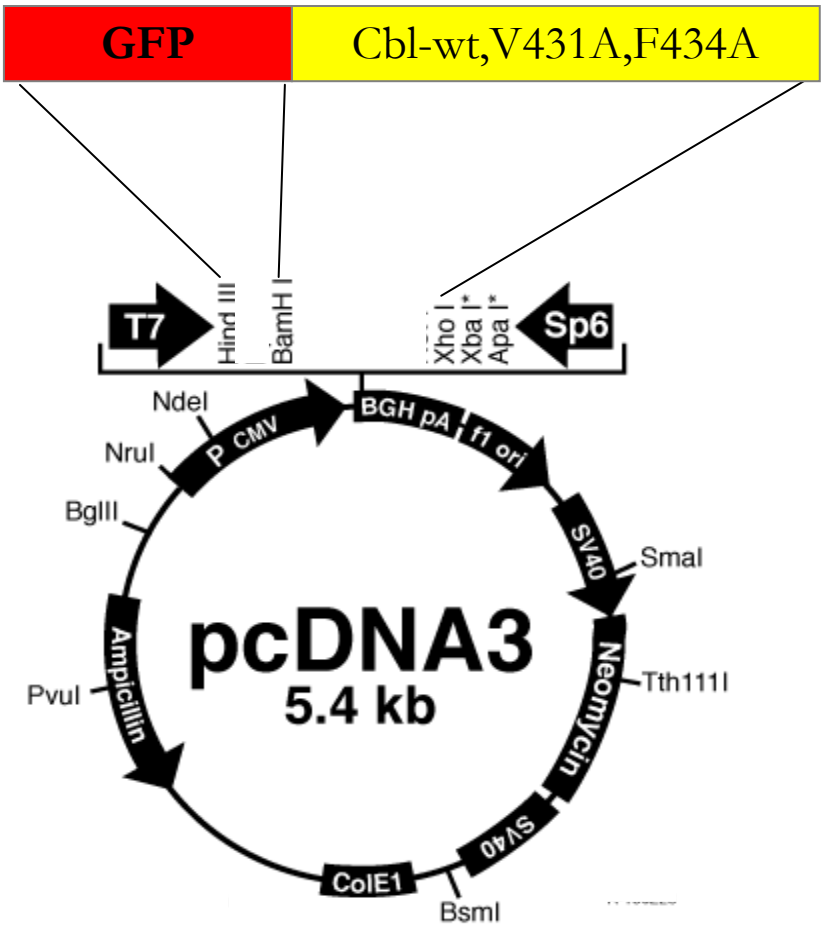


Figure 6: Representation of the donor plasmids that provide inserts for cloning. The Lill lab provided all of the donor plasmids. These included: pcDNA3-2xStop, pcDNA3-GFP-Cbl-N, -wt, -V431A, and -F434A.

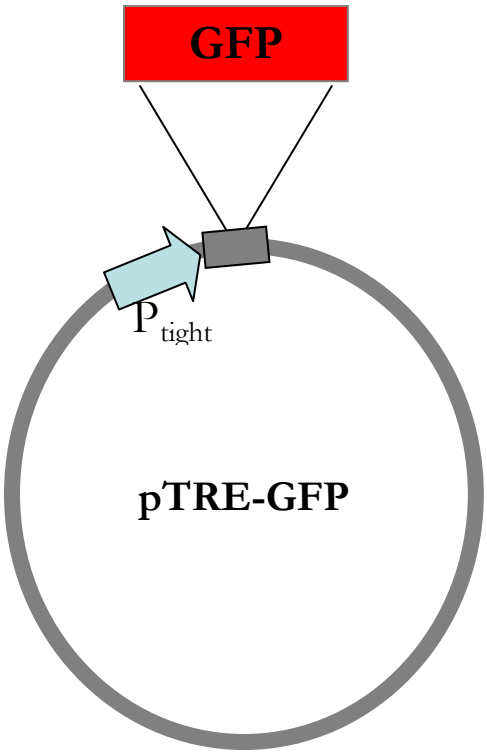


Figure 7: pTRE-Tight-GFP-2xStop, the first construct generated.

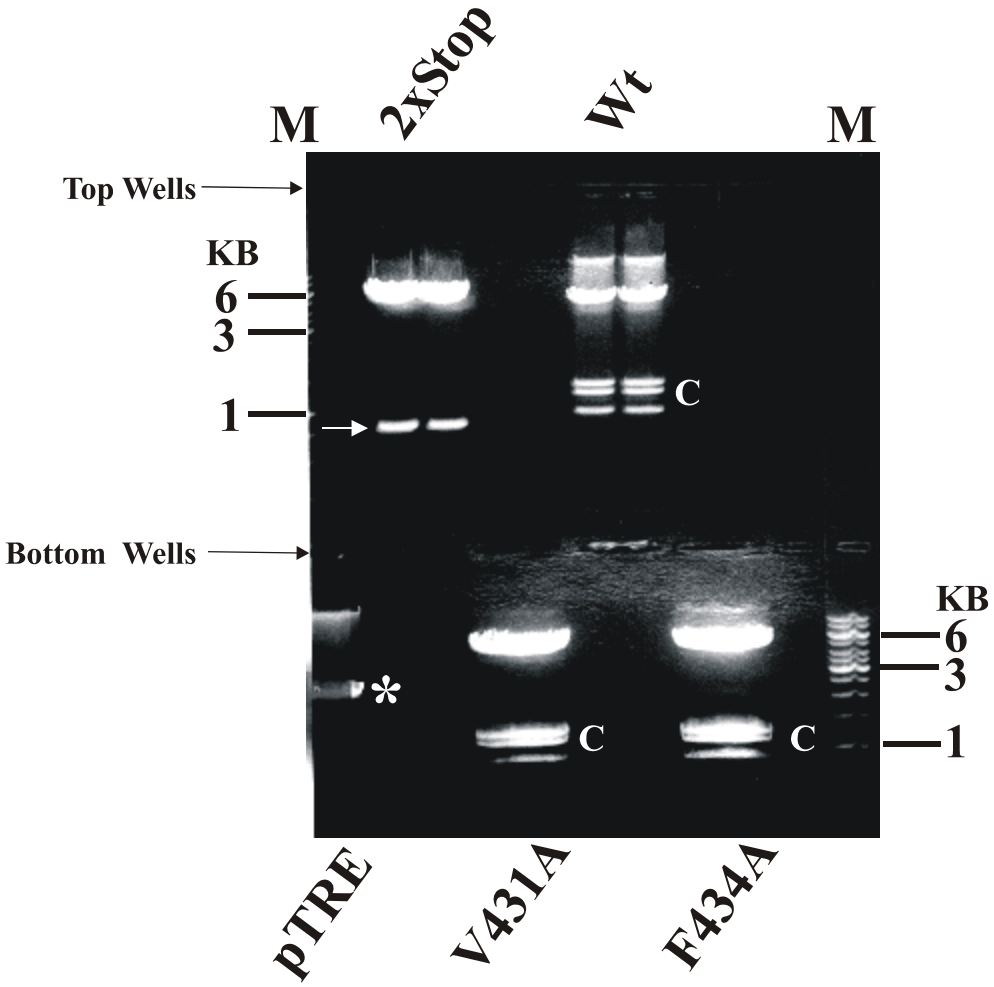


Figure 8. Preliminary characterization of the plasmids pcDNA3-GFP-2xStop, pcDNA3-GFP-Cbl-wt, pcDNA3-GFP-Cbl-V431A, pcDNA3-GFP-Cbl-F434A, and the cloning vector pTRE-Tight. Plasmid samples, all treated with the restriction endonucleases HindIII and XbaI, were separated on a 1.0% agarose/TAE gel. The intact GFP coding insert is indicated by the white arrow; the linearized pTRE cloning vector is indicated by the asterisk; and the multiple-digested Cbl coding sequences are indicated by a C. The size of the linear DNA markers is indicated at the far left and far right of the panel, in kilobase pairs. It was determined that there are multiple HindIII restriction sites in the Cbl coding sequence, making all but the pcDNA3-2xStop and pTRE Tight constructs unsuitable for digestion fragment exchange using these sites.

To generate the pTRE-GFP construct, large amounts of HindIII/XbaI-treated pTRE-tight and pcDNA3-2xStop were separated by gel electrophoresis (Figure 9). The now-linear pTRE vector and the GFP insert were then purified, ligated to each other, and then used to transform *E. coli* cells. To ensure that the correct plasmid had been generated, multiple clones were characterized through digestion and gel electrophoresis (Figure 10). Of the five clones characterized, four were deemed adequate based on the size of the DNA fragments. The sample represented in lane 6 from Figure 10 was chosen for further use in the project.

We determined that the most effective solution for generation of the remaining constructs was to create a parent construct with a new restriction site incorporated upstream of the genes of interest (Figure 11). The generation of the parent construct is described in the Plasmids section of Methods. Mutagenic PCR was carried out on pcDNA3-GFP-Cbl-N and products were separated on an agarose gel (Figure 12). Both the PCR product and the cloning vector pTRE-Tight were preparatively digested using the restriction enzymes KpnI and XbaI (Figure 13). The results of the cloning and purification of the new construct are characterized in Figure 14.

Our next goal was to create the experimental constructs: pTRE-GFP-Cbl-wt, pTRE-GFP-Cbl-V431A, and pTRE-GFP-Cbl-F434A. The general design of the experimental constructs is illustrated in Figure 15. The parent construct was preparatively digested using the restriction enzymes BamHI and XbaI, as were the donor constructs (Figure 16). After the ligation, transformation, and purification of the clones, gel electrophoresis was used to confirm the generation of the correct constructs (Figure 17).

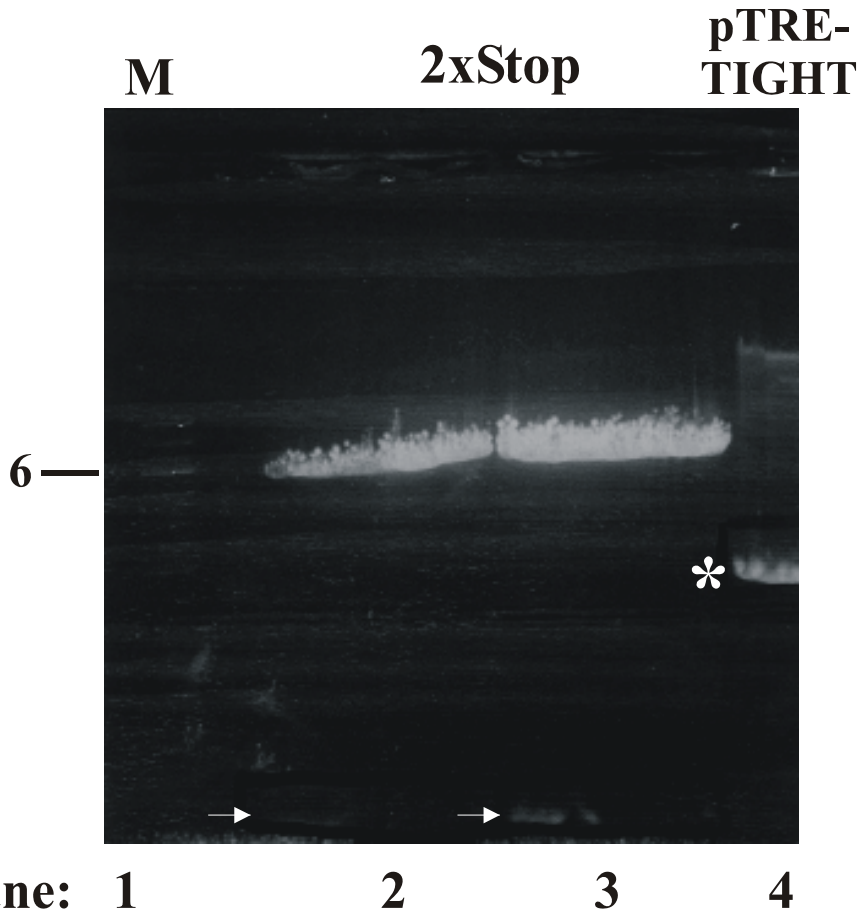


Figure 9. Preparative digestion of pcDNA3-2xStop and pTRE-Tight plasmids. Both samples were digested using the restriction enzymes HindIII and BamHI, followed by separation on a 1.0% agarose/TAE gel. The positions of the GFP inserts are indicated by white arrows; the position of linear pTRE DNA is indicated by a white asterisk. Both lanes 2 and 3 contain the pcDNA3-2xStop sample, while the pTRE digest is in lane 4. The size of the linear DNA markers loaded in lane 1 is indicated at the left of the panel, in kilobase pairs.

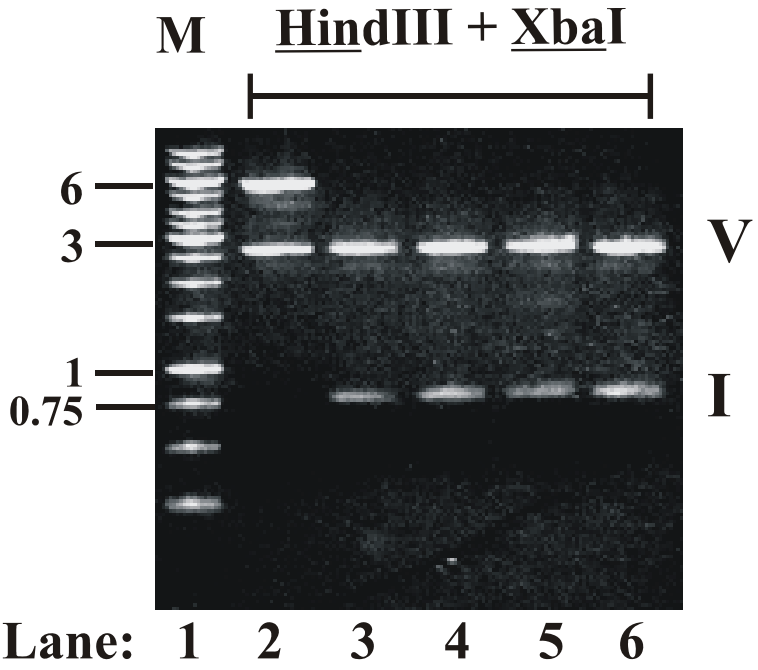


Figure 10. Characterization of the pTRE-GFP-2xStop plasmid construct. Upon ligation of the HindIII/XbaI-treated GFP insert and pTRE vector, bacterial cells were transformed. DNA minipreps were prepared from the resulting bacterial colonies using the QIAprep Spin Miniprep kit. The plasmid DNAs were screened by HindIII and XbaI digestion, followed by separation on a 1.0% agarose/TAE gel. The position of the linear insert DNA fragment is indicated by the letter I to the right of the panel; the position of the linear vector DNA fragment is indicated by the letter V to the right of the panel. The size of the linear DNA markers is indicated at the left of the panel, in kilobase pairs.

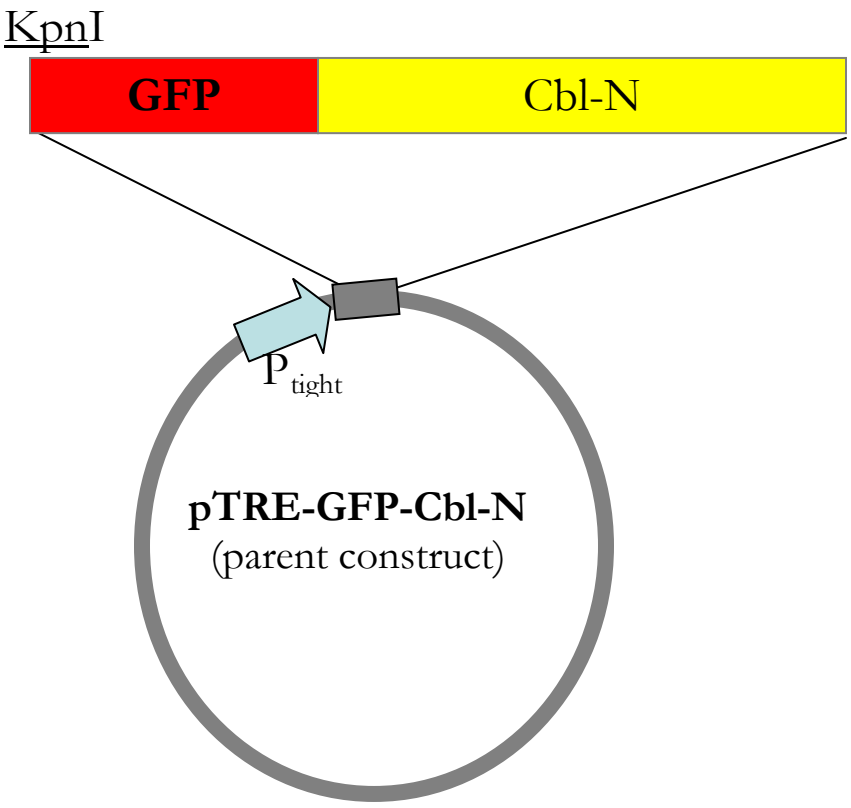


Figure 11: Representation of the parent pTRE construct for cloning of Cbl full-length sequences. The goal was to generate the parental construct pTRE-GFP-Cbl-N with a new KpnI restriction site just upstream of the GFP-Cbl-N region but down stream of the P_{tight} inducible promotor.

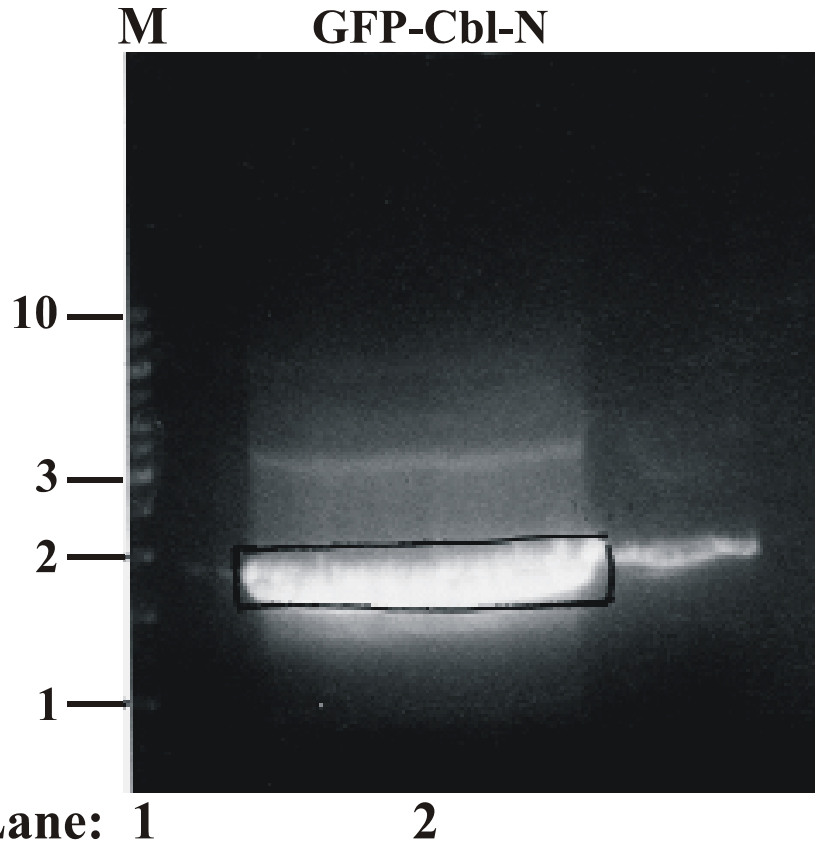


Figure 12. Characterization of GFP-Cbl-N PCR product to be used to generate parent cloning vector. Untreated PCR products (see Methods) were separated on a 1.0% agarose/TAE gel. The position of the linear DNA is indicated by the black outline The size of the linear DNA markers in lane 1 is indicated left of the panel, in kilobase pairs. The size of the fragment confirms this is the expected GFP-Cbl-N sequence, roughly 1.8kb. The upstream primer used in the PCR procedure, IA-193, created a KpnI restriction site just upstream of the GFP-coding sequence. The downstream primer, OH-019, maintained an XbaI restriction site just downstream of the Cbl-N coding sequence.

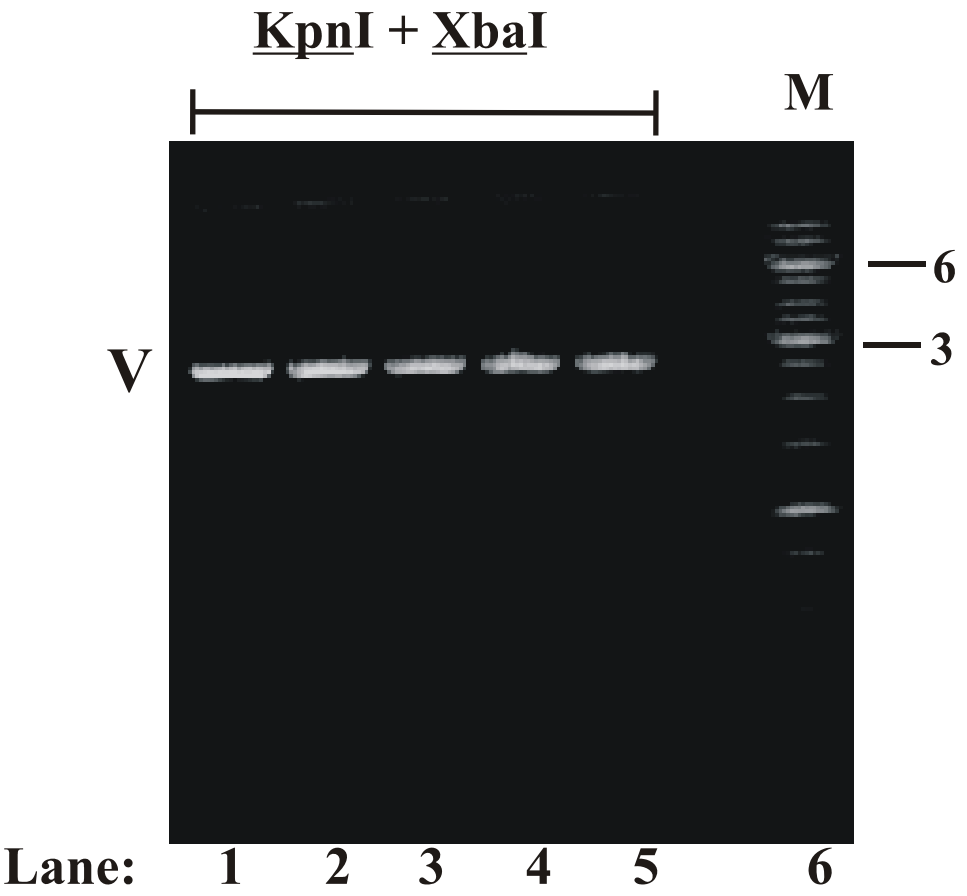


Figure 13. Digestion of cloning vector in preparation for ligation. The cloning vector pTRE-Tight was treated with restriction enzymes KpnI and XbaI, followed by separation on a 1.0% agarose/TAE gel. The position of the linear DNA fragment is indicated by the letter V to the left of the panel. The size of the linear DNA markers in lane 6 is indicated at the right of the panel, in kilobase pairs.

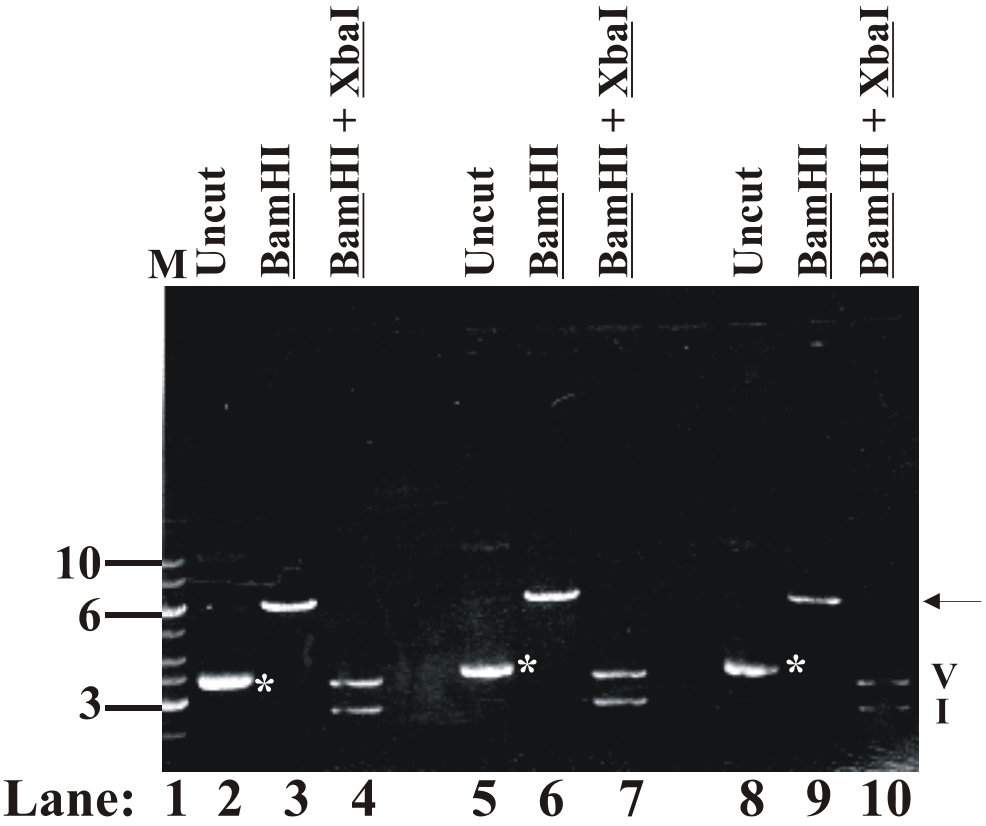


Figure 17. Confirmation that Cbl-wt, -V431A, and -F434A inserts were correctly cloned into pTRE. Untreated, single-digested and double-digested plasmid samples were separated on a 1.0% agarose/TAE gel. The positions of the intact, linear, and fragmented DNAs are indicated by the following symbols: white asterisk, uncut; filled arrow, linear; V, vector fragment; I, insert fragment. The size of the lane 1 linear DNA markers is indicated at the left of the panel, in kilobase pairs.

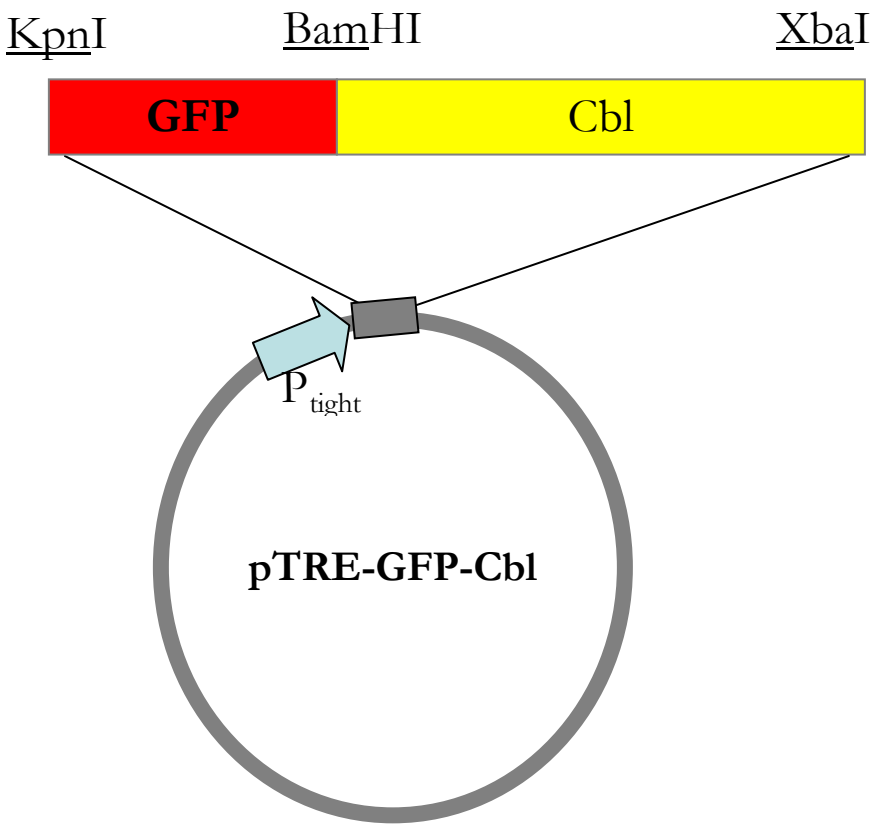


Figure 15. Cloning sites used for insertion of Cbl sequences into the parent vector to generate experimental constructs. The goal was to generate the constructs pTRE-GFP-Cbl-wt, -V431A, and -F434A from the parent construct pTRE-GFP-Cbl-N.

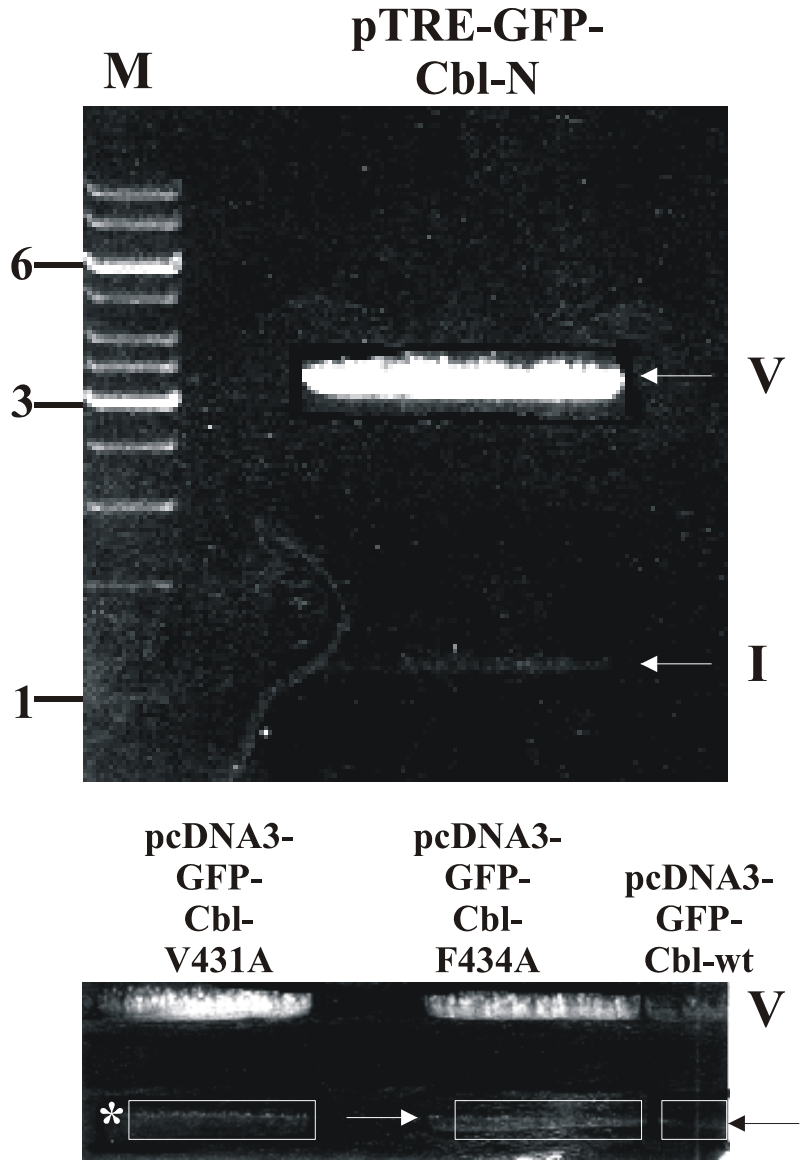
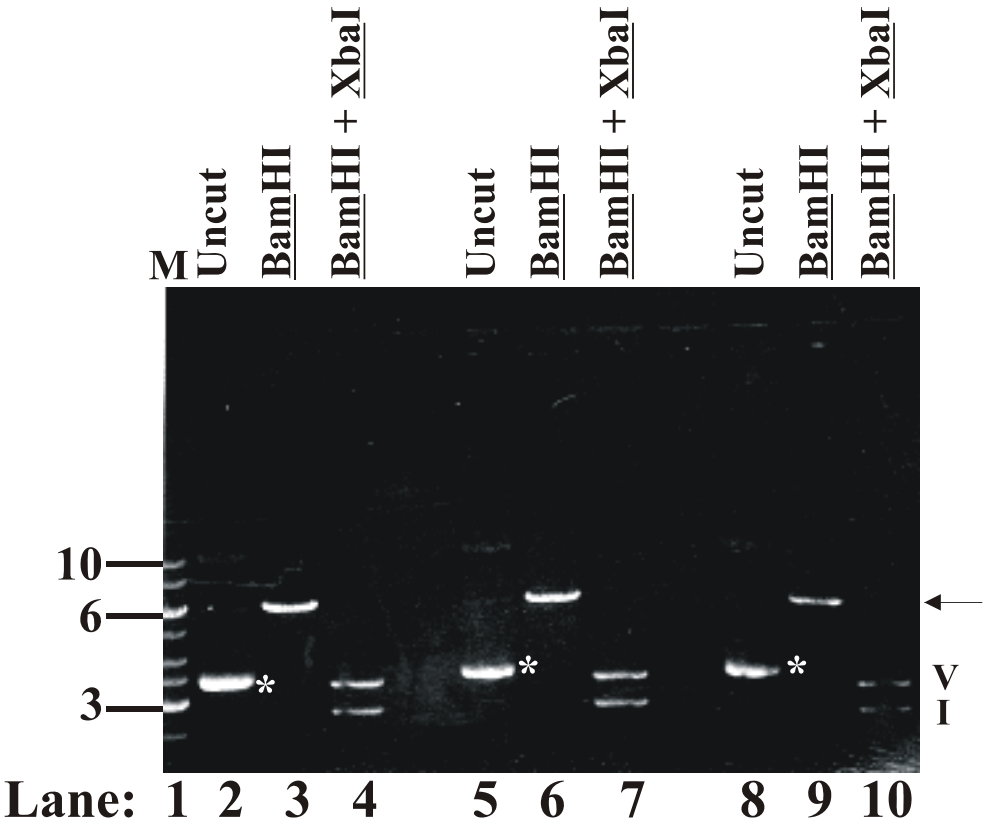


Figure 16. Preparative digestion of parent vector construct and insert donor constructs to generate fragments for cloning. **Top Panel**: Digestion of the parent construct, pTRE-GFP-Cbl-N. The parent construct was digested with the restriction enzymes BamHI and XbaI, removing the Cbl-N insert. The restriction endonuclease-treated fragments were then separated on a 1.0% agarose/TAE gel. The size of the linear DNA markers is indicated at the left of the panel, in kilobase pairs. The liberated Cbl-N insert is indicated by the letter I, along with a white arrow. The linearized pTRE-GFP fragment is indicated by the letter V, along with a white arrow as well. **Bottom Panel**: Digestion of pcDNA3-GFP-Cbl-wt, -V431A, and -F434A with BamHI and XbaI. The digested fragments were gel-separated. The liberated linear DNA inserts are indicated by white boxes and the following symbols: white asterisk, Cbl-V431A; white arrow, Cbl-F434A; black arrow, Cbl-wt.



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Once all of the constructs had been produced, the ensuing step was to transfect HEK 293 TET-On Advanced mammalian cells for transient expressor studies. This was carried out for the purpose of testing the inducibility of the constructs in the presence of the antibiotic doxycycline. As shown in Figure 18, the addition of 100 ng/mL of doxycycline to the transfected cells resulted in the expression of the genes immediately downstream from the P_{tight} promotor on each plasmid. Also important was the absence of any signal in the non-stimulated samples, confirming the tightness of the system. In all, this procedure showed that the P_{tight} promotor is indeed doxycycline-induced.

A second transient transfection was carried out in order to determine the optimum concentration of doxycycline necessary for induction of the promotor, while limiting toxicity to cells. The results of this procedure, known as a dose response assay, are depicted in Figure 19. Concentrations of doxycycline ranging from 0 to 750 ng/mL were added to transiently transfected cells. Cell proteins were harvested and analyzed by Western blotting using an antibody against GFP. The sizes of the protein signals in the top panel of Figure 19 correspond well with the known size of the GFP-Cbl-wt fusion protein – approximately 147 kD. The strength of the bands increases as the concentration of doxycycline increases, up to 250 ng/mL. Beyond this concentration, the bands are about equal in appearance. In addition to this, concentrations of doxycycline exceeding 100 ng/mL seemed to have negative effects on the cells, which included the following: slow growth, morphological change, lack of adherence to the plate bottom, and cell death. Thus, it was determined that the optimal balance between toxicity and protein expression was achieved at a doxycycline concentration of 100 ng/mL.

To produce stable cell lines, the cells were transfected with the experimental constructs, as well as a hygromycin selection marker. Upon treatment with hygromycin, individual colonies were selected. GFP proteins were then induced by doxycycline treatment, harvested and analyzed by Western blotting using an antibody against GFP. Cell lines of the pTRE-GFP (Figure 20) and pTRE-GFP-Cbl-N (Figures 21 and 22) constructs were successfully produced. A stable cell line may also have been produced for the pTRE-GFP-Cbl-F434A mutant construct, as shown in lane 18 of Figure 22.

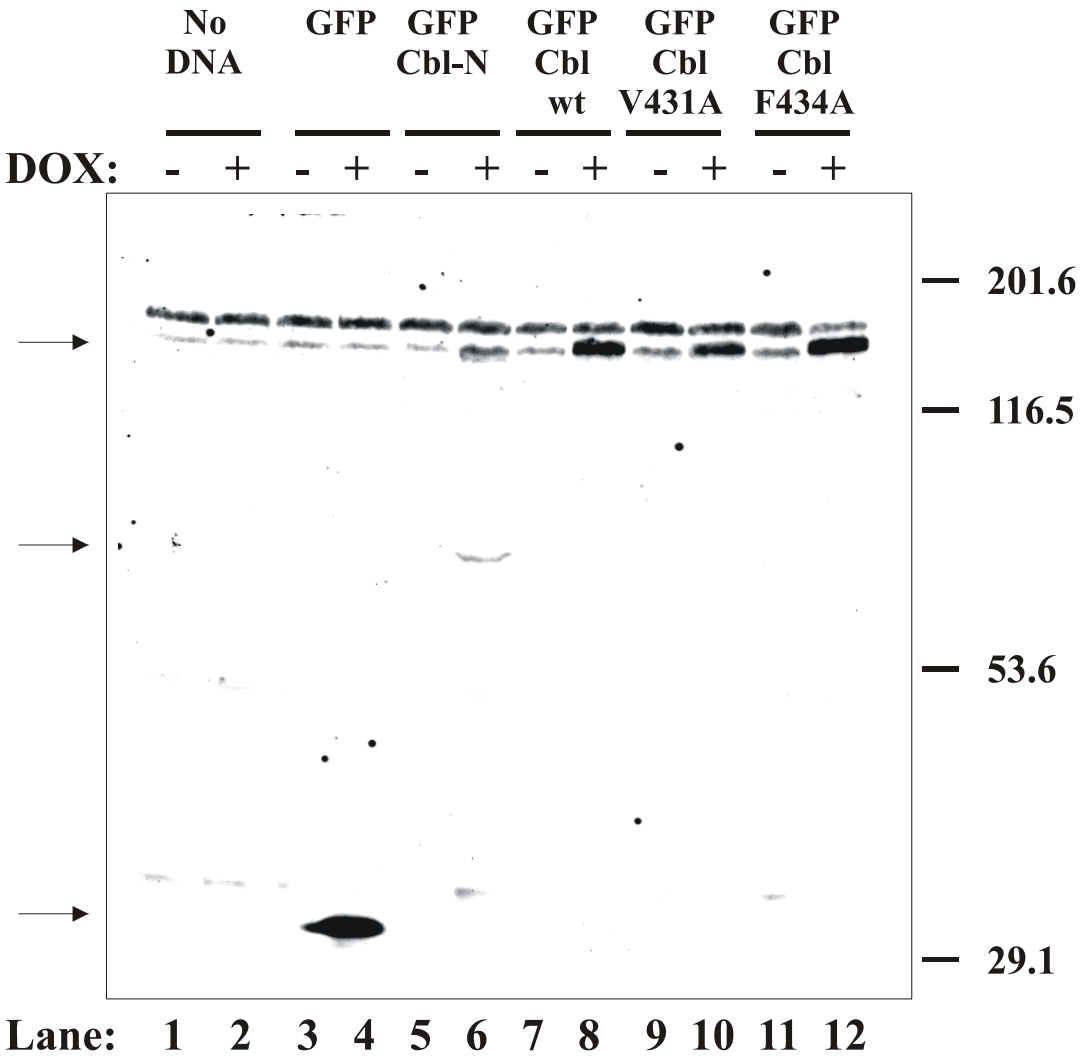

Figure 18. Transient, induced expression of GFP or GFP-Cbl fusion proteins in HEK 293 TET-ON Advanced cells. The cells were transfected with different constructs using the calcium phosphate precipitation method as described in Methods.The precipitate was left on for 12 to 16 hours. Approximately 24 hours after removal of precipitates from matched transfection plates, one plate received media containing 100ng/ml doxycycline; the other matched plate received media without doxycycline. After approximately 24 hours, all cultures were harvested for protein extraction as mentioned in the Methods sections. A total of 100 μg of cellular protein was loaded per lane of an 8% SDS-PAGE protein gel. The resolved proteins were then transferred to a PVDF membrane, which was probed with anti-GFP antibody, as detailed in the Methods section under immunoblotting. The three arrows to the left of the panel indicate the positions of the induced proteins.

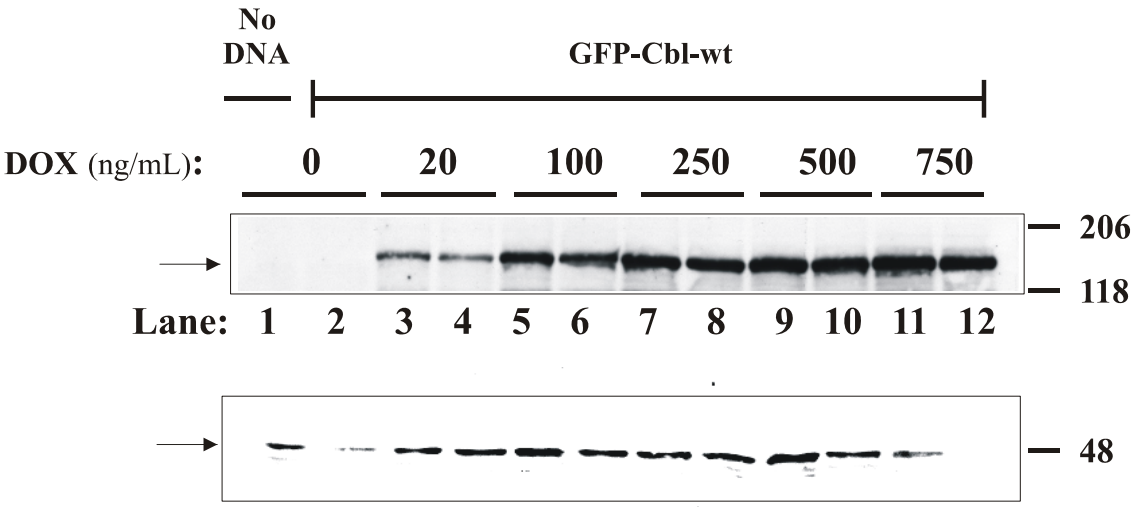

Figure 19. Transient, induced expression of GFP-Cbl-wt fusion proteins in HEK 293 TET-ON Advanced cells for the purpose of a dose response assay. The cells were transfected with the pTRE-GFP-Cbl-wt construct using the calcium phosphate precipitation method as described in Methods. The precipitate was left on for 12 to 16 hours. Approximately 24 hours after removal of precipitates, pairs of transfection plates received media containing varying concentrations of doxycycline, as indicated above the top panel. After approximately 24 hours, all cultures were harvested for protein extraction as mentioned in the Methods section. A total of 100 µg of cellular protein was loaded per lane of an 8% SDS-PAGE protein gel. The resolved proteins were then transferred to a PVDF membrane, which was probed first with an anti-GFP antibody [Top panel] then with an anti-gamma-tubulin antibody [Bottom], as detailed in the Methods section under immunoblotting. The arrow to the left of the top panel indicates the position of the induced proteins; the arrow to the left of the bottom panel indicates the position of the gamma-tubulin protein. One culture was untranfected in order to serve as a negative contro;, it is designated "No DNA" in the first lane of the top panel.

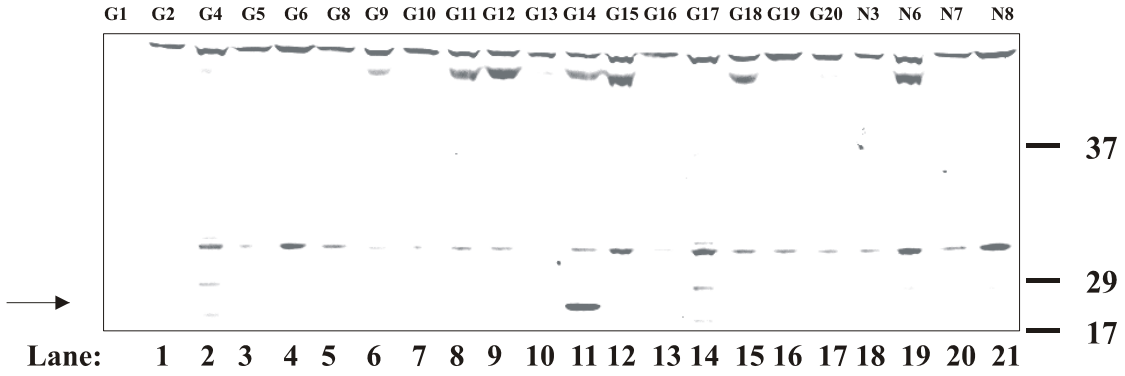

Figure 20. Induced expression of GFP and GFP-Cbl-N fusion proteins in HEK 293 TET-ON Advanced cells. The cells were transfected with different constructs using the calcium phosphate precipitation method as described in Methods. The precipitate was left on for 12 to 16 hours. Approximately 72 hours after removal of precipitates, 100 μg/mL of hygromycin was added, followed by subsequent additions of 100 μg/mL doses of hygromycin approximately every four days. After approximately 3 weeks, media containing 100 ng/mL of doxycycline were added to the plates, followed by extraction of proteins for harvesting 24 hours later, as mentioned in the Methods section. A total of 100 μg of cellular protein was loaded per lane of an 10% SDS-PAGE protein gel. The resolved proteins were then transferred to a PVDF membrane, which was probed with anti-GFP antibody, as detailed in the Methods section under immunoblotting. The single arrow to the left of the panel indicates the position of the induced protein. Along the top of the figure are the designations given to the samples: those beginning with the letter "G" indicate the GFP protein, while "N" indicates the GFP-Cbl-N fusion protein.

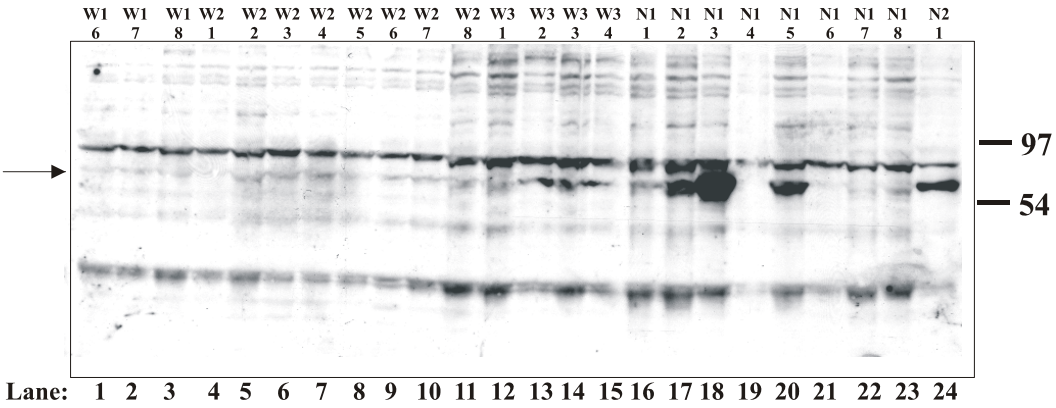

Figure 21. Induced expression of GFP-Cbl-wt or GFP-Cbl-N fusion proteins in HEK 293 TET-ON Advanced cells. The cells were transfected with different constructs using the calcium phosphate precipitation method as described in Methods. The precipitate was left on for 12 to 16 hours. Approximately 72 hours after removal of precipitates, 100 μg/mL of hygromycin was added, followed by subsequent additions of 100 μg/mL doses of hygromycin approximately every four days. After approximately 3 weeks, media containing 100 ng/mL of doxycycline were added to the plates, followed by extraction of proteins for harvesting 24 hours later, as mentioned in the Methods section. A total of 100 μg of cellular protein was loaded per lane of an 10% SDS-PAGE protein gel. The resolved proteins were then transferred to a PVDF membrane, which was probed with anti-GFP antibody, as detailed in the Methods section under Immunoblotting. The single arrow to the left of the panel indicates the position of the induced protein. Along the top of the figure are the designations given to the samples: those beginning with the letter "W" indicate the GFP-Cbl-wt protein, while "N" indicates the GFP-Cbl-N fusion protein. Lanes 17, 18, 20, and 24 have a single strong band between 54 and 97 kD, the approximate size of the truncation mutant Cbl-N fused to GFP, indicating potential positive clones for that construct.

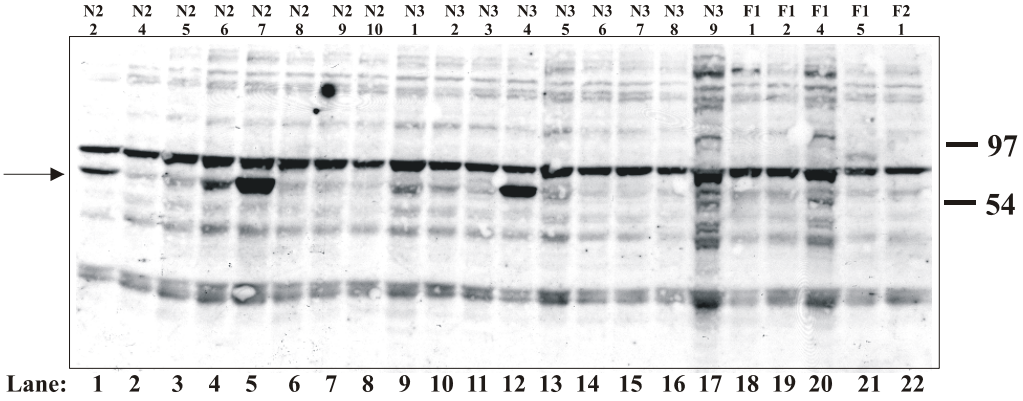

Figure 22. Induced expression of GFP-Cbl-wt or GFP-Cbl-N fusion proteins in HEK 293 TET-ON Advanced cells. The cells were transfected with different constructs using the calcium phosphate precipitation method as described in Methods. The precipitate was left on for 12 to 16 hours. Approximately 72 hours after removal of precipitates, 100 μg/mL of hygromycin was added, followed by subsequent additions of 100 μg/mL doses of hygromycin approximately every four days. After approximately 3 weeks, media containing 100 ng/mL of doxycycline were added to the plates, followed by extraction of proteins for harvesting 24 hours later, as mentioned in the Methods section. A total of 100 μg of cellular protein was loaded per lane of an 10% SDS-PAGE protein gel. The resolved proteins were then transferred to a PVDF membrane, which was probed with anti-GFP antibody, as detailed in the Methods section under Immunoblotting. The single arrow to the left of the panel indicates the position of the induced protein. Along the top of the figure are the designations given to the samples: those beginning with the letter "N" indicate the GFP-Cbl-N fusion protein while those beginning with an "F" indicate the GFP-Cbl-F434A protein . Lanes 1, 4, 5, and 12 have a single strong band between 54 and 97 kD, the approximate size of the truncation mutant Cbl-N fused to GFP, indicating potential positive clones for that construct. Lane 18 also has a very faint band at approximately the expected location for a for a full-length Cbl protein fused to GFP. This indicates a potential positive pTRE-GFP-Cbl-F434A clone.

Chapter IV: Discussion

This investigation has resulted in the generation of multiple plasmid constructs that can and will be used in the future by the Lill lab for the purposes of studying the effects of ectopically expressed wild type and mutant forms of Cbl on EGF-R signaling in a cellular context. The generation of the pTRE-GFP construct is significant because it will serve as a control for the effects of GFP on cellular functions, which is necessary since all of the other constructs will express GFP-Cbl fusion proteins. The pTRE-GFP-Cbl-N construct serves as a positive control for the induction of a cancer phenotype and oncogenic signaling. Cbl-N is a known transforming agent that has been shown to cause tumors in animals (8). It lacks the linker region, RING finger domain, and RING finger tail that have been shown to contribute to Cbl's evolutionarily conserved regulation of EGF-R (10, 15).

The pTRE-GFP-Cbl-wt construct will provide the reference level of signaling by the wild type Cbl protein, against which the full-length RF tail mutant forms will be compared. The two mutant constructs, pTRE-GFP-Cbl-V431A and pTRE-GFP-Cbl-F434A, will allow us to determine the effects of two specific mutations in the RF tail region of the Cbl protein on EGF-R signaling. The Lill laboratory has already shown that these mutations compromise EGF-R degradative trafficking (16). Subsequent signaling studies will address the question of whether changes in receptor tyrosine kinase trafficking lead to changes in receptor signaling, either at the cell surface or on internalized endosomes.

Signaling by the Cbl RF tail mutants will be analyzed by Western blotting. The lysates to be tested will come from matched sets of unstimulated and EGF-stimulated

cultures that have been induced with doxycycline to express a wt or mutant Cbl protein. The first signaling pathway to be tested will be the PLC-γ pathway, which depends upon the presence of the EGF-R at the cell surface. I will compare the V431A-induced signal against the signal from wt Cbl, quantifying both the total PLC-γ and phospho-PLC-γ levels. I predict that the signaling through this pathway will remain elevated for significantly longer time periods in V431A cells than in cells expressing wild type Cbl, since internalization of EGF-R is blocked by this mutant.

With the pTRE-GFP-Cbl-F434A mutant, on the other hand, we expect PLC-γ signaling levels to be comparable to wild type cells, since this mutant does not affect internalization of EGF-R. However, we predict ligand-dependent increases in MAP kinase and Akt signaling (which have been suggested to occur both at the plasma membrane and inside of the cell on endosomes) with a longer duration in the F434A mutant than in wild type cells, because degradation of activated EGF-R in F434A-expressing cells is blocked at the level of endosome fusion after internalization has occurred (16). This could allow EGF-R to continuously initiate signaling even from the limiting membrane of endosomes.

I have succeeded in generating stable cell lines that inducibly express proteins from two of the constructs that I developed for my thesis project (pTRE-GFP and pTRE-GFP-Cbl-N), and possibly for pTRE-GFP-Cbl-F434A as well (Figure 22, lane 18). This has laid the ground work for the signaling experiments mentioned above. The use of stable inducer cell lines, as opposed to transiently transfected cells, will mitigate the issue of low transfection rates, since all cells in a given clonal cell line will theoretically possess and express the plasmid of interest. The use of inducible Cbl expressors also will

mitigate a problem that was noted in previous studies: continuous expression of the signaling suppressor Cbl selects for the outgrowth of stable expressor lines that have mutated to bypass Cbl's function (2). The inducible lines should not have this problem if they are maintained in the absence of doxycycline.

After we have established clonal inducible cell lines for the remaining three plasmids and carried out the signaling experiments in vitro, all of the control and RF mutant pTRE-tight Cbl constructs can be utilized further for transgenic mouse experiments. Expression of the desired proteins can be induced by adding doxycycline to the food or water of the transgenic mice, which can then be monitored for evidence of oncogenic signaling. Tumorigenesis is expected with the GFP-Cbl-N construct, and it may also be caused by the V431A and F434A mutants.

In summary, using the RF tail mutant expression constructs, the Lill lab will now be able to undertake studies of Cbl trafficking and signaling in the context of intact mammalian cell cultures and in transgenic mice. The resulting data will show whether the Cbl RF tail, which controls the endocytic trafficking of EGF-R, also is a key regulator of the receptor's oncogenic signaling.

References

1. **Beguinot, L., R. M. Lyall, M. C. Willingham, and I. Pastan.** 1984. Down-regulation of the epidermal growth factor receptor in KB cells is due to receptor internalization and subsequent degradation in lysosomes. Proc Natl Acad Sci U S A **81:**2384-8.
2. **Bonita, D. P., S. Miyake, M. L. Lupher, Jr., W. Y. Langdon, and H. Band.** 1997. Phosphotyrosine binding domain-dependent upregulation of the platelet-derived growth factor receptor alpha signaling cascade by transforming mutants of Cbl: implications for Cbl's function and oncogenicity. Mol Cell Biol **17:**4597-610.
3. **Brandt, R., R. Eisenbrandt, F. Leenders, W. Zschiesche, B. Binas, C. Juergensen, and F. Theuring.** 2000. Mammary gland specific hEGF receptor transgene expression induces neoplasia and inhibits differentiation. Oncogene **19:**2129-37.
4. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456-67.
5. **Gullick, W. J.** 1998. Type I growth factor receptors: current status and future work. Biochem Soc Symp **63:**193-8.
6. **Kim, H., and W. J. Muller.** 1999. The role of the epidermal growth factor receptor family in mammary tumorigenesis and metastasis. Exp Cell Res **253:**78-87.
7. **King, A. C., and P. Cuatrecasas.** 1981. Long-term epidermal growth factor-receptor internalization and processing in quiescent human fibroblasts. J Supramol Struct Cell Biochem **17:**377-87.
8. **Langdon, WY, Hartley, JW, Klinken, SP, Ruscetti, SK, Morse, HC III.** PNAS U.S.A. 1989 86(4):1168-1172
9. **Lill, N. L., P. Douillard, R. A. Awwad, S. Ota, M. L. Lupher, Jr., S. Miyake, N. Meissner-Lula, V. W. Hsu, and H. Band.** 2000. The evolutionarily conserved N-terminal region of Cbl is sufficient to enhance down-regulation of the epidermal growth factor receptor. J Biol Chem **275:**367-77.
10. **Miettinen, P. J., J. E. Berger, J. Meneses, Y. Phung, R. A. Pedersen, Z. Werb, and R. Derynck.** 1995. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. Nature **376:**337-41.
11. **Miyake, S., K. P. Mullane-Robinson, N. L. Lill, P. Douillard, and H. Band.** 1999. Cbl-mediated negative regulation of platelet-derived growth factor receptor-dependent cell proliferation. A critical role for Cbl tyrosine kinase-binding domain. J Biol Chem **274:**16619-28.
12. **Stern, K. A., G. D. Visser Smit, T. L. Place, S. Winistorfer, R. C. Piper, and N. L. Lill.** 2007. Cbl enhances epidermal growth factor receptor degradation by modulating Hrs tyrosine phosphorylation. Mol Cell Biol **27:**888-898, published online November 13, 2006.
13. **Stoscheck, C. M., and G. Carpenter.** 1984. Down regulation of epidermal growth factor receptors: direct demonstration of receptor degradation in human fibroblasts. J Cell Biol **98:**1048-53.

14. **Sun, W., Q. Yan, T.A. Vida, and A.J. Bean.** 2003. Hrs regulates early endosome fusion by inhibiting formation of an endosomal SNARE complex. J Cell Biol. **162**(1): 125-37.
15. **Visser, G. D., and N. L. Lill.** 2005. The Cbl RING finger C-terminal flank controls epidermal growth factor receptor fate downstream of receptor ubiquitination. Exp Cell Res **311:**281-93.
16. **Visser Smit, G.D., T.L. Place, S.L. Cole, K. A. Clausen, S. Vemuganti, G. Zhang, J.G. Koland, and N.L. Lill.** 2009. Cbl Controls EGFR Fate by Regulating Early Endosome Fusion. Science Sig. 2: 1-8.